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LysoPC activates the Akt pathway to up-regulate ECM protein production in human aortic valve cells

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Abstract

Background—Overproduction of extracellular matrix (ECM) protein by aortic valve interstitial cells (AVICs) plays an important role in valvular sclerosis (thickening) associated with the early pathobiology of aortic stenosis. Accumulation of oxidized low-density lipoprotein (oxLDL) is observed in sclerotic aortic valve and may have a mechanistic role in valvular disease progression. Lysophosphatidylcholine (LysoPC) is a component of oxLDL and has multiple biological activities.

Objective—This study was to test the hypothesis that oxLDL and LysoPC up-regulate ECM protein production in human AVICs.

Methods and Results—AVICs were isolated from normal human aortic valves. Cells were treated with oxLDL (40 µg/ml) or LysoPC (40 µmol/L). Immunoblotting was applied to analyze ECM proteins (collagens I, III and biglycan) in cell lysate and Picrosirius red staining is used to examine collagen deposition. Both oxLDL and LysoPC up-regulated the production of biglycan and collagen I. The up-regulation of ECM proteins by LysoPC was preceded by the phosphorylation of Akt and ERK1/2. Inhibition of Akt markedly reduced the effect of LysoPC on ECM protein production and collagen deposition. However, inhibition of ERK1/2 had no effect.

Conclusion—LysoPC up-regulates the production of biglycan and collagen I in human AVICs and may mediate the effect of oxLDL on ECM protein production. The Akt pathway appears to be critical in mediating the effect of LysoPC. OxLDL accumulation and generation of LysoPC in the

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Author contributions

H.C., Q.Y., R.S. and Y.Z. performed the experiments and analyzed data. H.C. drafted the manuscript. D.A.F. and W.W. discussed the experimental results and revised the manuscript. X.M. conceived and designed the experiments, supervised the entire study and finalized the manuscript.

Disclosures

All authors disclose no financial and personal relationships with other people or organizations that could potentially and inappropriately influence (bias) their work and conclusions.

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aortic valve tissue may contribute to the mechanism of valvular sclerosis associated with the development and progression of aortic stenosis.

Keywords

Lysophosphatidylcholine; oxidized low-density lipoprotein; Akt; extracellular matrix protein; aortic valve

Introduction

Aortic stenosis involves valvular sclerosis and calcification (1). Aortic valve sclerosis (AVS), defined as leaflet thickening without obstruction of left ventricular outflow, occurs in a large number of people age 65 years and older and is an early sign of the development of aortic stenosis (1). Although moderate AVS does not cause left ventricular outflow obstruction, it is associated with an increased risk of cardiovascular death and myocardial infarction (2). Thus, suppression of the process of AVS will reduce the risk for associated cardiovascular conditions and prevent the development of severe aortic stenosis. Understanding the cellular and molecular mechanisms of AVS is required for pharmacological suppression of the development and progression of AVS.

Increasing evidence shows that AVS is an active process that has a similarity to the pathobiological process of atherosclerosis, including lipoprotein deposition, chronic inflammation and extensive remodeling of extracellular matrix (3, 4). Oxidized low-density lipoprotein (oxLDL) has the pro-inflammatory and growth-stimulating properties (5), and is involved in the pathobiology of atherosclerosis (6). Several studies have found oxLDL accumulation in sclerotic aortic valves (7). Our previous studies found that oxLDL up-regulates the expression of bone morphogenetic protein-2 (BMP-2) in human aortic valve interstitial cells (AVICs) (8, 9) and human coronary artery endothelial cells (10). Furthermore, oxLDL synergizes with lipopolysaccharide to elicit augmented AVIC osteogenic responses (11). It appears that oxLDL has an impact on human AVIC pro-osteogenic activity. Currently, the effect of oxLDL and its components on the production of extracellular matrix (ECM) proteins by aortic valve cells is unclear although over-production of ECM proteins contributes to the pathobiology of AVS.

Lysophosphatidylcholine (LysoPC) is the main phospholipid component of oxLDL. It is a hydrolysis product of phosphatidylcholine (PC) in oxLDL (12). In vertebrate plasma, increased levels of LysoPC have been found in atherosclerotic vascular tissue. LysoPC can induce the pro-osteogenic responses in vascular smooth muscle cells and AVICs (13, 14). Thus, it possesses a similar activity as oxLDL.

Growing evidence shows that the accumulation of ECM proteins is a characteristic change in AVS (15). Human AVICs, primarily fibroblast-like cells and myofibroblasts, are a population of resident cells in the aortic valve tissue and actively synthesize ECM proteins, including biglycan (BGN, a stationary component of the ECM), collagen I, collagen III and collagen IV (15, 16). Currently, the effect of oxLDL and LysoPC on the production of ECM proteins in human AVICs is unclear.

We hypothesized that oxLDL is capable of elevating human AVIC activity to overproduce ECM proteins. The purpose of this study was to determine: 1) the effect of oxLDL ECM protein production in human AVICs, 2) whether LysoPC may mimic the effect of oxLDL in human AVICs, 3) whether oxLDL and LysoPC influence AVIC production of ECM proteins by inducing myofibroblastic transition and 4) what signaling mechanism up-regulates ECM protein production in human AVICs.

Materials and Methods

Materials

Antibody against biglycan was purchased from R&D System (Minneapolis, MN). Antibodies against Collagen I and Collagen III were purchased from Abcam (Cambridge, MA). Antibodies against α -smooth muscle actin (α -SMA), phosphorylated Akt (Ser473 or Thr 308), total Akt, phosphorylated ERK1/2 and total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Antibody against β -actin was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Medium 199 was purchased from Lonza (Walkersville, MD). ERK1/2 inhibitor PD98059 was purchased from EMD Millipore (Billerica, MA). MK2206, a specific Akt inhibitor, was purchased from Selleckchem (Houston, TX). OxLDL was purchased from Alfa Aesar (Lancashire, UK). LysoPC and all other chemicals and reagents were purchased from Sigma-Aldrich Chemical (St Louis, MO).

Cell isolation and culture

This study was approved by the Colorado Multiple Institutional Review Board of the University of Colorado Denver. Normal aortic valve leaflets were collected from the explanted hearts of 6 heart transplant recipients (4 males and 2 females; mean age, 59 ± 8.1 years) with cardiomyopathy. The valve leaflets were thin and had no histological abnormality. All patients gave informed consent for the use of their aortic valves for this study.

AVICs were isolated and cultured using the previously described method (17) with modifications (18). Briefly, valve leaflets were subjected to an initial digestion with a high concentration of collagenase (2.5 mg/ml) to remove endothelial cells. Then, the remaining tissue was treated with a low concentration of collagenase (0.8 mg/ml) to free the interstitial cells. Cells were collected by centrifugation and cultured in M199 growth medium containing penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum. AVIC isolates obtained by using this protocol are a lack of endothelial cells as verified by von Willebrand factor staining. Each isolate from a separate donor is used as a cell line. Thus, 6 normal AVIC cell lines were used in this study. Cells of passages 3 to 6 were treated when they reached 80% to 90% confluence.

To determine the effect of oxLDL on the production of α -SMA, biglycan, collagen I and III, cells were treated with oxLDL (40 μ g/ml) for 24 to 72 hours. LysoPC from egg yolk was dissolved in ethanol to make a stock solution. To determine the effect of LysoPC on the production of these proteins, cells were treated with LysoPC (20 or 40 μ mol/L; 7.1 or 14.2 μ g/ml; final concentration of ethanol being 0.12 or 0.24%) for 24 or 48 hours. Protein levels

of α -SMA, biglycan, collagen I and collagen III in cell lysates were assessed by immunoblotting. Cells treated with 40 μ mol/L of LysoPC for 1 to 8 hours were used to analyze the phosphorylation of ERK1/2 and Akt. To determine the role of the ERK1/2 and Akt pathways in modulation of ECM protein production, cells were treated with ERK1/2 inhibitor (PD98059, 25 μ mol/L) or Akt inhibitor (MK2206, 5.0 μ mol/L) 1 hour prior to the exposure to LysoPC. In additional experiments, AVICs were treated with LysoPC (40 μ mol/L) for 7 days with or without Akt inhibitor. Picrosirius red staining (PSR) was applied to evaluate the role of the Akt pathway in mediating collagen deposition.

Immunoblotting

Immunoblotting was applied to analyze the levels of α -SMA, biglycan, collagen I, collagen III, phosphorylation of ERK1/2, total ERK1/2, phosphorylation of Akt and total Akt, with β -actin as the loading control. Cells were lysed in a sample buffer (100 mmol/L Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol). Protein samples were separated on gradient (4%–20%) mini-gels and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were blocked with 5% skim milk solution for 1 hour at room temperature. Then, the blocked membranes were incubated with a primary antibody against the protein of interest. After washing with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Sigma-Aldrich Chemical, St Louis, MO), the membranes were incubated with a peroxidase-linked secondary antibody specific to the primary antibody applied. After further wash, membranes were treated with enhanced chemiluminescence reagents. The membranes were exposed on x-ray films. The Image J software (Wayne Rasband, National Institutes of Health, Bethesda, MD) was used to analyze protein band density.

Picrosirius red staining

Picrosirius red staining of cultured cells can detect collagen deposition and has been applied as a tool for analysis of in vitro fibrogenesis (19). We applied Picrosirius red staining to examine collagen deposition. In brief, cells on 96-well plates were treated with LysoPC in the presence or absence of inhibitor. At the end of the experiment, cells were fixed in ice-cold methanol overnight at -20°C , washed with PBS and then incubated with 0.1% Picrosirius red (0.5 mg/ml in saturated aqueous solution of picric acid) for 2 hours. Following rinses with 0.1% acetic acid, plates were air-dried and examined by microscopy. PSR stain was examined and photographed with a Nikon Eclipse TS100 microscope (Tokyo, Japan).

Statistical Analysis

All data are presented as mean $\pm$ standard error (S.E.). Statistical analysis was performed using StatView software (Abacus Concepts, Calabasas, CA). One-way analysis of variance (ANOVA) with the post hoc Fisher test was used to analyze differences between multiple groups. Student's t-test was used for comparison between two groups. Nonparametric Mann-Whitney U test was performed to confirm the difference of the 2 group comparison. For multiple group comparisons, nonparametric Kruskal-Wallis test was performed to confirm the differences. Statistical significance was defined as $P < 0.05$.

Results

OxLDL up-regulates the production of biglycan and collagen I

We analyzed the levels of biglycan, collagen I and collagen III in cell lysates after treatment with oxLDL. As shown in Figure 1, treatment of AVICs with oxLDL for 48 or 72 hours increased the levels of biglycan and collagen I. While oxLDL induced a moderate increase in collagen III levels in human AVICs, the changes were not significant (Figure 1).

LysoPC also up-regulates the production of biglycan and collagen I

To evaluate the effect of LysoPC on ECM protein production in human AVICs, we treated AVICs with LysoPC for 24 and 48 hours. As shown in Figure 2, treatment of with 20 or 40 $\mu\text{mol/L}$ of LysoPC induced similar increases in the levels of biglycan and collagen I at 24 or 48 hours. It appears the concentrations of LysoPC applied has comparable effect on ECM protein production in human AVICs.

OxLDL and LysoPC have no effect on α -smooth muscle actin (α -SMA) level

To examine whether oxLDL and LysoPC promote AVIC transition from a fibroblast-like phenotype to a myofibroblastic phenotype, we analyzed the levels of α -SMA, a biomarker of myofibroblast. Figure 3 show that neither oxLDL nor LysoPC has a significant effect on α -SMA levels in human AVICs. Thus, oxLDL and LysoPC increase ECM protein production in human AVICs not though induction of myofibroblastic transition.

LysoPC induces rapid phosphorylation of ERK1/2 and Akt

Since myofibroblastic transition is not involved in the mechanism by which LysoPC up-regulates ECM protein production, we examined whether LysoPC activates cellular signaling pathways. Following an exposure to LysoPC (40 $\mu\text{mol/L}$) for 1–8 hours, phosphorylation of ERK1/2 and Akt Ser⁴⁷³ was evident (Figure 4). Phosphorylation of Akt Thr³⁰⁸ was also observed (not shown). Phosphorylation of these two kinases occurred at 1 hour and remained elevated at 2 hour. However, the levels of phospho-p38 MAPK were unchanged at all time points following an exposure to LysoPC (data not shown). Thus, LysoPC activates the ERK1/2 and Akt pathways in human AVICs.

LysoPC up-regulates ECM protein production in human AVICs through the Akt pathway

To determine the relative role of the ERK1/2 and Akt pathways in mediating LysoPC-induced biglycan and collagen I production, we applied specific inhibitors of ERK1/2 and Akt. Figure 5A shows that inhibition of ERK1/2 failed to affect the up-regulation of biglycan and collagen I production by LysoPC. In contrast, inhibition of Akt markedly reduced the effect of LysoPC on biglycan and collagen I production (Figure 5B). Therefore, the Akt pathway, rather than the ERK1/2 pathway, mediates the up-regulation of ECM protein production induced by LysoPC in human AVICs.

To confirm the ability of LysoPC in up-regulation of ECM protein production and the regulatory role of Akt, we applied Picosirius red staining to examine collagen deposition in human AVICs after a prolonged exposure to LysoPC. The images in Figure 5C show that LysoPC induced collagen deposition and that inhibition of Akt reduced collagen deposition

in cells exposed to LysoPC. Therefore, a prolonged exposure to LysoPC causes greater collagen deposition in human AVIC culture and the Akt pathway plays an important role in mediating the “pro-fibrogenic” effect of LysoPC.

Discussion

Valvular ECM remodeling is a dynamic process and occurs constantly in response to shear stress. Maladaptive valvular ECM remodeling occurs following injury and noxious stimulation and contributes to the mechanism underlying AVS development and progression, resulting in thickened and stiffened valvular leaflets that eventually obstruct blood flow and compromise cardiac function (20).

Valvular ECM consists of a matrix of collagens, elastins, proteoglycans, and glycoproteins (15, 16). ECM protein production depends on AVIC activity. In the present study, we observed that oxLDL and LysoPC, a phospholipid of oxLDL, are capable of up-regulating the production of biglycan and collagen I in human AVICs. While LysoPC does not change the levels of myofibroblastic biomarker α -SMA, it activates the Akt pathway to up-regulate collagen I and biglycan production and collagen deposition. As oxLDL accumulation is associated with AVS, we speculate that oxLDL accumulation and generation of LysoPC in the aortic valve tissue may contribute to the mechanism underlying ECM protein over-production and valvular fibrosis associated with AVS development and progression.

Biglycan, a member of the family of small proteoglycans, is a stationary component of the valvular ECM (21). Several studies have reported biglycan accumulation in sclerotic areas of stenotic human aortic valves (8, 22). Moreover, previous studies from our group have found that AVICs of stenotic human aortic valves express higher levels of biglycan than cells of normal valves and that soluble biglycan is potent to up-regulate the production of BMP-2, alkaline phosphatase and pro-inflammatory cytokines in human AVICs (8, 23). Therefore, AVIC overproduction of biglycan caused by oxLDL or LysoPC may elevate the levels of soluble form of this ECM protein, leading to aortic valve inflammation and calcification. Collagen I and III are components of fibrils that provide tissues with tensile strength and influence cell attachment and migration. Collagen I is the most abundant ECM protein in the aortic valve (16). Collagen I and III are present in valve cusps with a ratio of 3:1, and over-production of these two collagens occurs in response to injury and other stimuli. Up-regulation of collagen I without a significant change in collagen III production in human AVICs exposed to oxLDL will alter the ratio of collagen I and III in valvular tissue. This may result in adverse ECM remodeling that has an impact on valvular function.

OxLDL accumulation in sclerotic or stenotic aortic valves has been reported by a number of studies (4, 24). Higher levels of oxLDL in the aortic valve is associated with increased tissue remodeling score, suggesting that oxLDL may be involved in the fibrosis and calcification of the aortic valve. LysoPC mimics several intracellular signaling events triggered by whole oxLDL (25). OxLDL increased protein levels of biglycan and collagen I in human AVICs at 48 hours, whereas treatment with LysoPC for 24 hours was sufficient to increase biglycan and collagen I production. The temporal difference may be due to the distinct biological

activities of oxLDL and LysoPC. In this regard, the active groups in oxLDL may need to be exposed or LysoPC needs to be released from oxLDL before an effect can be exerted.

AVICs are heterogeneous. However, greater than 90% of cells in AVIC isolates from normal human aortic valves are fibroblasts, and the remaining cells are myofibroblasts (18, 26). It is well known that myofibroblasts have greater activities and express higher levels of ECM proteins (15). Expression of higher levels of α -SMA are characteristic for myofibroblasts. To understand whether oxLDL or LysoPC induce AVIC phenotypic transition from fibroblasts to myofibroblasts, we analyzed cellular α -SMA levels following treatment of cells with oxLDL or LysoPC. However, neither oxLDL nor LysoPC altered α -SMA levels. The results of this study indicate that oxLDL and LysoPC activate human AVICs to increase the production of biglycan and collagen I without causing a cell phenotypic change. It is likely that cellular signaling mechanisms that regulate the production of these two ECM proteins are involved.

Several signaling pathways, including the ERK1/2, Akt and p38 MAPK pathways, modulate AVIC activities (15, 27, 28). In this regard, inhibition of p38 MAPK and ERK1/2 has been shown to block TGF- β -mediated expression of biglycan in vascular smooth-muscle cells (29) and Akt is involved in the expression of collagens in the mesangium and renal tubulo-
interstitium cells (30). Further, LysoPC has been found to induce the activation of ERK1/2 and p38 MAPK in vascular smooth muscle cells (31, 32) and to activate Akt in human vascular endothelial cells (33). We observed in this study that LysoPC induced a rapid activation of ERK1/2 and Akt, but had no effect on the phosphorylation of p38 MAPK. The results from the experiments using blockades show that inhibition of ERK1/2 has no effect on the up-regulation of biglycan and collagen I production by LysoPC although LysoPC activates this protein kinase. In contrast, inhibition of Akt essentially abrogated the up-regulation of biglycan and collagen I production and collagen deposition induced by LysoPC in human AVIC cultures. Thus, Akt, rather than ERK1/2, plays a critical role in mediating LysoPC-induced ECM protein production in human AVICs. It is possible that the Akt pathway play is involved in valvular ECM remodeling induced by oxLDL and LysoPC.

It should be noted that a cross-talk between the Akt and ERK1/2 signaling pathways exists (34). However, the results of the present study indicate that Akt mediates ECM protein production in human AVICs through an ERK1/2-independent mechanism even if both Akt and ERK1/2 are activated. Our previous studies demonstrate that ERK1/2 is involved in the up-regulation of pro-osteogenic protein BMP-2 by oxLDL in human AVICs and human coronary artery endothelial cells (9, 10). It appears that the ERK1/2 pathway plays an important role in modulation of the pro-osteogenic response to oxLDL in cardiovascular cells.

It remains unclear from this study whether oxLDL and LysoPC exert the effect extracellularly or intracellularly and what receptor is responsible if an extracellular action is involved. In addition, the role of cytokines in the up-regulation of ECM protein production by oxLDL and LysoPC has not been determined although several reports show that oxLDL and LysoPC are capable of modulating cytokine production in macrophages and vascular endothelial cells (35, 36). Future studies are needed to address these issues. Furthermore, all

results of this study are obtained from in vitro experiments. Translation of the findings to an in vivo condition should be done with caution.

Conclusion

LysoPC up-regulates the production of biglycan and collagen I in human AVICs and may mediate the effect of oxLDL on ECM protein production. The Akt pathway plays a critical role in mediating the effect of LysoPC on ECM protein production and collagen deposition. OxLDL accumulation and generation of LysoPC in the aortic valve tissue may contribute to the mechanism of valvular sclerosis associated with the development and progression of aortic stenosis.

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Figure 1. OxLDL up-regulates the production of biglycan and collagen I in human AVICs
 AVICs from normal human aortic valves were treated with oxLDL (40 µg/ml) for 24–72 hours. Representative immunoblots and densitometric data show significant elevation of biglycan and collagen I production, but not collagen III production, by oxLDL. Data is presented as the mean ± S.E. of 5 different experiments using distinct AVIC isolates.
 * $P < 0.05$ vs. control.

A

Figure 2. LysoPC up-regulates the production of biglycan and collagen I in human AVICs
 Human AVICs were treated with LysoPC (20 or 40 $\mu\text{mol/L}$) for 24–48 hours. Control cells were treated with equal concentrations of vehicle (0.12 and 0.24% of ethanol). Representative immunoblots and densitometric data show that LysoPC markedly increases the production of biglycan and collagen I. Data is presented as the mean $\pm$ S.E. of 5 different experiments using distinct AVIC isolates. * $P < 0.05$ vs. control.

Figure 3. OxLDL and LysoPC have no effect on α -smooth muscle actin levels in human AVICs
 Human AVICs were treated with oxLDL (40 μg/ml) for 24–72 hours or with LysoPC (20 or 40 μmol/L) for 24–48 hours. Control cells were treated with equal concentrations of vehicle (0.12 and 0.24% of ethanol). Representative immunoblots of 3 separate experiments show that neither oxLDL nor LysoPC has an effect on α -SMA levels.

Figure 4. LysoPC induces the phosphorylation of Akt and ERK1/2 in human AVICs

Human AVICs were treated with LysoPC (40 $\mu\text{mol/L}$) for 1–8 hours. Control cells were treated with equal concentrations of vehicle (0.12 and 0.24% of ethanol). Representative immunoblots of 3 separate experiments show that LysoPC induces the phosphorylation of Akt and ERK1/2 at 1 and 2 hours. P = phospho; T = total.

Figure 5. Inhibition of Akt suppresses LysoPC-induced production of biglycan and collagen I and collagen deposition in human AVICs

A. AVICs were exposed to LysoPC (40 μ mol/L) for 24 hours in the presence or absence of a specific ERK1/2 inhibitor (PD98059 25 μ mol/L). Representative immunoblots of 3 separate experiments show that inhibition of ERK1/2 has no effect on biglycan and collagen I production in cells exposed to LysoPC. **B.** AVICs were exposed to LysoPC (40 μ mol/L) for 24 hours in the presence or absence of a specific Akt inhibitor (MK2206, 5.0 μ mol/L). Representative immunoblots and densitometric data shows that inhibition of Akt reduces biglycan and collagen I levels in cells exposed to LysoPC. N=5 separate experiments using distinct AVIC isolates. * P <0.05 vs. control; # P <0.05 vs. LysoPC alone. **C.** AVICs were exposed to LysoPC (40 μ mol/L) for 7 days in the presence or absence of Akt inhibitor. Representative images (10 $\times$ objective) of 3 separate experiments show that collagen deposition detected by Picrosirius red staining is increased following treatment with LysoPC. Inhibition of Akt reduces LysoPC-induced collagen deposition.